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# Relationships between antral follicle count, blood serum concentration of anti-Müllerian hormone and fertility in mares

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# 1 Zusammenfassung

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Zusammenhänge zwischen der Anzahl ovarieller Antralfollikel, der Blutserumkonzentration des Anti-Müller-Hormons und der Fertilität von Stuten

Die Ziele dieser Studie waren es, Zusammenhänge zwischen der Antralfollikelzahl (AFZ), der Konzentration des Anti-Müller-Hormons (AMH) im Blutserum, dem Alter und der Fertilität von Stuten zu untersuchen. Insgesamt wurden 132 Stuten (Alter: 12.9 ± 4.5 Jahre; 3 bis 23 Jahre) sonographisch vor der künstlicher Besamung untersucht. Zeigten sie einen Follikel ≥35 mm und ein endometriales Ödem, wurde die Ovulation mit 3000 IE hCG i.v. induziert und zum gleichen Zeitpunkt eine Blutprobe zur Analyse von AMH entnommen. Zusätzlich wurden mittels Ultraschall alle Follikel auf beiden Ovarien gezählt, ihr Durchmesser bestimmt und anhand dessen klassifiziert (2-5, 6-10, 11-15, 16-20, 21-25, 26-30, 31-35, 35-40, >40 mm). Die Fertilität wurde anhand der saisonalen Trächtigkeitsrate (STR) beurteilt. Die AFZ lag zwischen 4 und 59 (Medianwert: 30) und die AMH-Konzentration zwischen 0.07 und 3.56 ng/ml (Mittelwert: 0.59 ng/ mL). Die AFZ von kleineren (Ø 2-30 mm: $r = 0.58$, $p < 0.0001$), aber nicht von grösseren Follikeln (Ø >30 mm: $r = 0.04$, $p > 0.05$) korrelierte mit der AMH-Konzentration. Weder die AFZ noch die AMH korrelierte ($p > 0.05$) mit dem Alter und der STR der Stuten. Zusammenfassend wiesen sowohl die AFZ als auch die AMH-Konzentration grosse individuelle Unterschiede auf. Die Zahl kleinerer, aber nicht diejenige grösserer Follikel korrelierte mit der AMH-Konzentration. Die AFZ und die AMH-Konzentration waren nicht altersabhängig und hatten keine Auswirkungen auf die Fertilität der Stuten.

Schlüsselwörter: Anti-Müller-Hormon, Antralfollikelzahl, Ovar, Fertilität, Pferd

# 2 Abstract

Vetsuisse Faculty Zurich (2018)

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Relationship between antral follicle count, blood serum concentration of anti-Müllerian hormone and fertility in mares

The objectives of this study were to determine relationships between antral follicle count (AFC), serum concentration of anti-Müllerian hormone (AMH), age and fertility in mares. In total, 132 brood mares (age: 12.9 ± 4.5 yrs; 3 to 23 yrs) were examined before AI by using transrectal ultrasonography. As soon as the mares developed a follicle with a mean diameter of ≥35 mm and endometrial oedema, ovulation was induced with 3000 IU hCG i.v. At the same time a blood sample was collected for the analysis of AMH. Additionally, follicles on both ovaries were counted, measured and classified according to their diameter (2-5, 6-10, 11-15, 16-20, 21-25, 26-30, 31-35, 35-40, >40 mm) by using ultrasonography. Fertility was estimated by seasonal pregnancy rate (SPR). The AFC ranged from 4 to 59 with a median value of 30 and AMH concentrations ranged from 0.07 to 3.56 ng/mL with a median value of 0.59 ng/mL. The AFC of smaller (Ø 2-30 mm: $r = 0.58$, $P < 0.0001$), but not of bigger follicles (Ø >30 mm: $r = 0.04$; $P > 0.05$) was related to AMH. Neither AFC nor AMH were associated with the age of the mares ($P > 0.05$). AFC and AMH did not affect SPR ($P > 0.05$). In summary, there was a high variability in antral follicle count as well as in anti-Müllerian hormone concentration between mares. The AMH concentration was only related with AFC of smaller, but not bigger follicles. Neither AMH nor AFC were related to age and fertility of the mares.

Keywords: anti-Müllerian hormone, antral follicle count, ovary, fertility, equine

# 3 Introduction

The number of follicles in female ovaries established during the fetal period is very variable among individuals in different species. The number varies between 350,000 and 1,100,000 in women (Forabosco et al., 1991; Gougeon et al., 1994), and between 14,000 and 250,000 in cows (Erickson, 1966a, b). In mares it ranges from 5,600 to 75,000 (Driancourt et al., 1982) and is distinctly lower than the number of follicles noticed in women and cows.

The number of follicles at birth serves for the entire reproductive period of each female and is never replenished (Gougeon, 1996; te Velde et al., 1998; te Velde, 1993). Only a restricted number (a few hundreds) of follicles reaches the growing pool and eventually ovulates. The rest becomes atretic by apoptosis (Hsueh et al., 1994). The process of atresia begins already during the fetal period and the number of follicles declines exponentially until the menopause in women. At the time of birth there are millions of follicles present and at the time of the menopause only hundreds or thousands of follicles are left (Richardson et al., 1987). A similar decrease is seen in domestic animals (Cushman et al., 2009; Erickson, 1966b; Ginther et al., 2009).

The ovarian reserve is defined as the number and quality of oocytes inside the ovarian follicles at a given time. Because of their small size (micrometer) the number of primordial follicles cannot be measured by clinical methods (Griffin et al., 2006). The precise amount of the ovarian reserve must be counted microscopically *post mortem* for an exact quantification (Block, 1952). A small proportion of primordial follicles develop to antral follicles, which are fluid-filled with a diameter ≥2 mm. These structures can be visualized by transvaginal (Meldrum et al., 1984; Pache et al., 1990) or transrectal sonography (Ginther and Pierson, 1984; Ireland et al., 2007). Their number is directly related to the number of primordial follicles (Gougeon, 1984; Hansen et al., 2011).

The anti-Müllerian hormone (AMH) is a good endocrine marker for the ovarian follicular reserve in women (Gruijters et al., 2003; Visser and Themmen, 2005; Visser et al., 2006), cows (Monniaux et al., 2010; Rico et al., 2009), mares (Vernunft et al., 2011), goats (Monniaux et al., 2011) and mice (Kevenaar et al., 2006). It is a glycoprotein, which is expressed only in the gonads (Cate et al., 1986). More precisely, in females it is exclusively synthesized in granulosa cells of small growing follicles (bovine: Monniaux et al., 2008; Vigier et al., 1984; ovine: Bezard et al., 1987;

human: Rajpert-De Meyts et al., 1999; Weenen et al., 2004). The concentration of AMH is high in small antral follicles and declines during terminal growth and atresia of the follicles (Baarends et al., 1995; Ball et al., 2008; Claes et al., 2015; Monniaux et al., 2008; Rico et al., 2009). It shows only small changes during the estrous cycle and throughout a time period of several months (Rico et al., 2009). The AMH is a good indicator of the follicular activity and is positively associated with antral follicle count (AFC) in cows (Ireland et al., 2008; Rico et al., 2009), as in humans (de Vet et al., 2002; Fanchin et al., 2003). Both parameters, AMH as well as AFC, can be used as quantitative markers of the ovarian reserve (Ireland et al., 2008; Kevenaar et al., 2006; van Rooij et al., 2005).

Several studies in women (Chang et al., 1998; Hendriks et al., 2005; Scheffer et al., 1999; Scheffer et al., 2003) and cows (Ireland et al., 2007; Mossa et al., 2012; Singh et al., 2004) have shown that AFC could give important information about fertility. For women it is now generally believed that fertility depends mainly on quantity and quality of the resting pool of oocytes, which is called the ovarian hypothesis. As the age of females has a significant effect on oocyte quality, embryos from premenopausal women have lower developmental rates compared to those from young women (Armstrong, 2001; Navot et al., 1991). This observation indicates that the endometrium is not as important as oocyte quality concerning fertility of premenopausal women (Cohen et al., 1999; Sauer et al., 1993).

The high variability of AFC among individuals with the same age can result from differences in nutrition of their mothers around the time of conception and during early pregnancy. Restricted maternal nutrition during the first trimester has a negative impact on the AFC in the offspring of cattle (Mossa et al., 2013) and sheep (Borwick et al., 1997; Da Silva et al., 2002; Rae et al., 2001). A chronic disease of pregnant cows can also affect reproductive performance of the daughters by lowering their follicle number. Dairy cows with high somatic cell counts (SCC) in milk for at least 4 to 5 times (>200,000 SC/mL) between two months before artificial insemination (AI) and parturition gave birth to daughters with a diminished ovarian reserve and, as a consequence, lower fertility, compared to cows that had not more than once an elevated SCC during this time period (Ireland et al., 2010). Furthermore, it was demonstrated that E. coli lipopolysaccharide (LPS) exerts a negative effect on the ovarian follicular reserve. *Ex vivo* studies on bovine ovaries as well as *in vivo* studies

on murine ovaries showed that the presence of LPS led to a decrease of the primordial follicle pool (Bromfield and Sheldon, 2013).

Up to now only a few studies have been published about AFC and AMH in mares. The concentration of AMH is constant during the estrous cycle and the different stages of gestation; however, it differs among mares (Almeida et al., 2011; Claes et al., 2015). A positive correlation between AFC and AMH could also be noticed in mares (Claes et al., 2015; Vernunft et al., 2011). The correlation between age of the mares and these parameters is discussed controversially. While no relationship was detected in mares aged between 10 and 21 years (Vernunft et al., 2011), those parameters were strongly correlated in older mares between 19 and 27 years (Claes et al., 2015).

There are distinct differences in the fertility of mares. A high age of mares is one of the most important causes for subfertility (Allen et al., 2007; Bosh et al., 2009; Lane et al., 2016), but other factors such as reproductive status (barren, maiden, lactating) (Allen et al., 2007; Sanderson and Allen, 1987) or breeding management also affect pregnancy rates (Bosh et al., 2009; Nath et al., 2010; Sieme et al., 2003). Mares used for breeding are often old, as they in general have been used in sport beforehand. The main reasons for subfertility in mares are pathological alterations of the uterus (Carnevale and Ginther, 1992; Doig et al., 1981; Jeffcott et al., 1982; Kenney, 1978). The persistent breeding-induced endometritis is considered to be the most frequent cause for disturbances in mare fertility (Carnevale and Ginther, 1992; LeBlanc and Causey, 2009; Ricketts and Alonso, 1991; Watson, 2000). However, other reasons than uterine failure such as defective oocytes can also have a negative effect on mare fertility (Ball et al., 1989; Carnevale et al., 1993).

To the best of our knowledge no studies on the effect of the ovarian follicle number on mare fertility have been published up to now. Therefore, the main objective of this study was to investigate if there are relationships between the antral follicle count, serum concentrations of AMH, and fertility in mares.

# 4 Material and methods

The whole experiment was performed in accordance with the animal use protocol of the Swiss animal protection society (protocol number 24726).

## 4.1 Mares

In total, 132 mares of different breeds (127 Warmblood, two Ponies, one Franches-Montagnes, one American Quarter Horse, one Arabian), presented for AI at a private Center of Equine Reproduction in Switzerland during the 2014 breading season (May-August), were used for the study. The mares were kept in straw-bedded boxes with permanent access to hay, grain and water, and were turned out daily to pasture. All mares were clinically healthy and had normal estrous cycles with a length of 18 to 23 days. According to their reproductive status the mares were divided in 4 different groups: maiden mares (MM), barren mares not inseminated during the last season (BNILS), barren mares inseminated during the last season (BILS) and lactating mares with a foal (LM).

## 4.2 Breeding management

Around the time of estrus the mares were examined daily by transrectal palpation and ultrasonography to determine the cycle stage and the day of ovulation. They were inseminated with fresh (n=20, 15%) and cryopreserved semen (n=112, 85%) from 86 different stallions.

When the mares showed distinct estrous signs (dominant follicle >35 mm, soft consistency, dilation of the cervix, endometrial oedema with endometrial folds appearing like the spokes of a wheel on cross-sectional ultrasound images), ovulation was induced with 3,000 I.U. hCG (Chorulon 5000 ad us. Vet., Veterinaria SA Freienbach, 8808 Pfäffikon, Switzerland). Just before the injection of hCG in the jugular vein, blood was collected passively through the inserted needle in a serum clot activator tube (Vacuette) for the analysis of Anti-Müllerian hormone in 131 mares. The collection of blood in one mare was inadvertently not performed.

In most of the mares AI was carried out 36 to 48 hours after hCG injection when the follicles showed a change in shape with a loss of their spherical appearance (Townson and Ginther, 1989) and a double wall on the ultrasound images (Pierson and Ginther, 1985). Two mares were not ovulating within 48 hours after the hCG injection. These animals were further monitored every 6 hours and inseminated again after ovulation. Due to restricted access to semen of 12 stallions, AI was carried out only within 6 hours after ovulation in 15 mares. Insemination was performed deeply into the uterine horn ipsilateral to the ovulatory follicle if frozen semen was used and into the uterine body if fresh semen was used.

After pre-ovulatory inseminations, ovulation was controlled within the next 12 hours. Furthermore, at this time the mares were controlled for intrauterine fluid accumulation and were treated according to a defined protocol (Knutti et al., 2000; Pycock and Newcombe, 1996; Troedsson et al., 1995). If intrauterine fluid with a diameter <2 cm was noticed (n=41), 10 I.U. oxytocin (Intertocin-S ad us. vet., MSD, Luzern, Switzerland) were injected i.m. If the accumulation was ≥2 cm (n=15), the uterus was flushed with 3 to 6 L of 0.9% NaCl until the fluid flushed back became transparent. The last flushing was followed by an intravenous injection of oxytocin. In both cases mares were re-examined 12-24 hours later and the therapy was adapted according to the findings (accumulation of fluids or not), using the same protocol as for the first examination after insemination.

Pregnancy was diagnosed 14-18 days after ovulation by ultrasonography. In case of a negative outcome, the mares were re-inseminated in the following cycle. In 15 mares, the stallions were changed during the breeding season to increase the probability of pregnancy by choosing a more fertile stallion. Fertility of the mares was estimated by seasonal pregnancy rate (SPR) and the number of cycles needed for a pregnancy (NCP).

## 4.3 Determination of antral follicle counts

To determine the total number of antral follicles, a transrectal ultrasonography was performed at the time of hCG-injection in 101 of the 132 mares. Two persons (HA and BK) examined the mares and made videos from the ovaries. Both ovaries were

examined systematically in sonographic 2D-mode (Esaote, Mylab 30 Vet Gold, Genoa, Italy) from the hilus to the ovulation fossa and videos with a duration of about 8 seconds were recorded. This examination and recording of the ovarian scans was repeated for 59 mares on the day of ovulation (Day 0) to investigate if the presence of pre-ovulatory follicles affected the counting of the follicles. All videos were evaluated off-line on a PC by one person (HA). As described by Claes et al. (2014), each follicle with a diameter ≥2 mm was counted and its largest diameter measured according to Ginther (1988). According to their diameter, the follicles were classified into different groups (2-5, 6-10, 11-15, 16-20, 21-25, 26-30, 31-35, 35-40, >40 mm).

## 4.4 Determination of serum AMH concentration

The blood samples were centrifuged (1200 x g for 10 min) immediately after collection and 0.5 mL serum were frozen (-19 °C) until analysis. Measurements were performed with the commercially available ELISA kit AMH Gen II A79765 from Beckman Coulter (Brea, California, USA) according to the instructions of the manufacturer. The intra- and inter-assay coefficients of variation for AMH were 4.5% and 5.5%, respectively. The limit of sensitivity of the AMH assay was 0.17 ng/mL.

## 4.5 Statistical analysis

Statistical analyses were conducted using the software StatView 5.0 (SAS Institute Inc., Cary, NC, USA). The distribution of the data was tested visually for normality and by means of the Kolmogorov-Smirnov test. The AFC values before and after ovulation were compared by using the Pearson correlation coefficient and the paired Student's t-test. Relationships between AFC and age of the mares were analysed by the Pearson correlation coefficient and the Fisher's r to z test and relationships between AMH and the age of the mares by using the Spearman Rank correlation. The AFC values of mares with different reproductive status and fertility were compared by using ANOVA and Fisher's PLSD test and the AMH values of mares with different reproductive status and fertility were compared with the Kruskal-Wallis

test. Data were presented as mean ± SD or median, minimum and maximum values as well as box plots showing median values, 25%, and 75% quartiles, 1.5 IQR and outliers. Differences were considered significant at $P \leq 0.05$.

# 5 Results

## 5.1 Age, reproductive status and fertility of the mares

The mean age of the mares was 12.9 ± 4.5 yrs (range: 3 to 23 yrs). Of the 132 mares, 22 (17%) were MM, 52 (39%) were LM, 30 (23%) were BNILS and 28 (21%) were BILS. At the end of the season, 98 mares were pregnant and 34 mares not (SPR = 74%). Mares were inseminated 1.76 ± 0.96 times (min. 1, max. 5) during the breeding season.

## 5.2 Antral follicle count and serum concentration of anti-Müllerian hormone

There was a high variability in the AFC between mares (Figure 1). The AFC decreased with the increasing size of the follicles (Table 1). In the mares, which were examined twice for the determination of the AFC, there was a high correlation (Figure 2) between both measurements ($r = 0.95$; $P < 0.05$), but the AFC on both ovaries was lower ($P < 0.05$) before ovulation (median 30; min. 4; max. 59) than after ovulation (median 30; min 2; max 61).

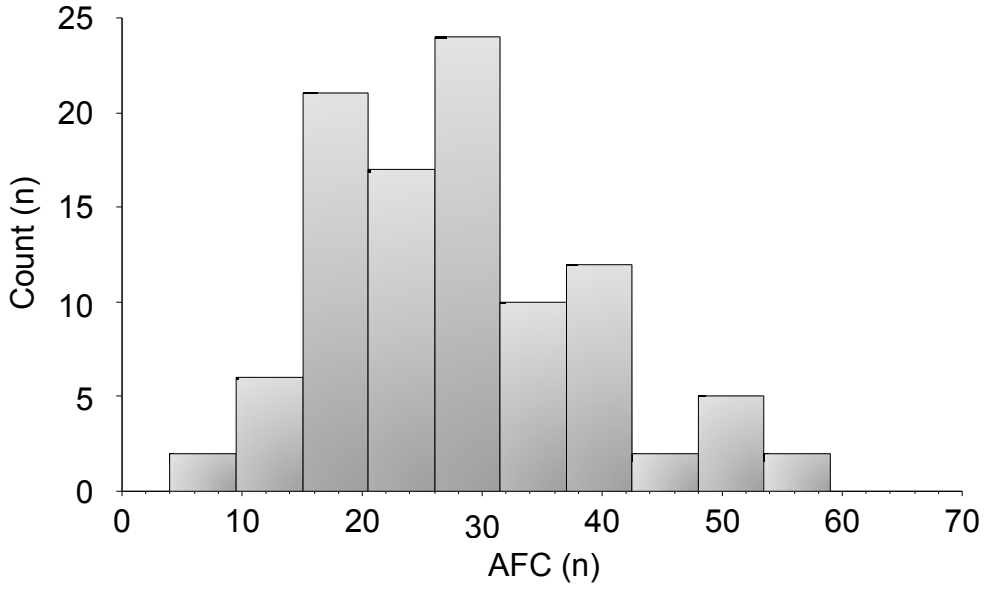


Figure 1: Histogram showing the frequency distribution of the number of antral follicles (AFC) in 132 mares.

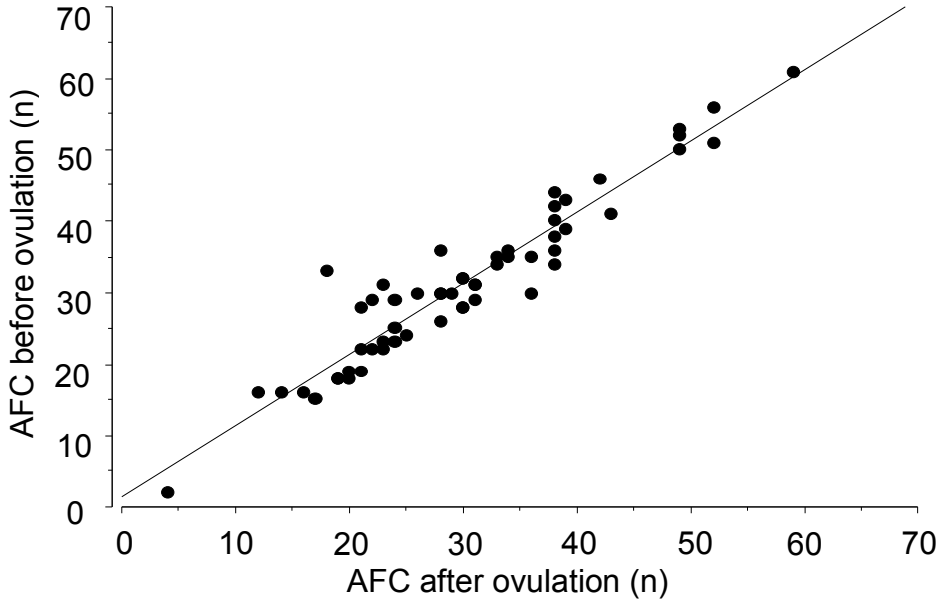


Figure 2: Relationship between the antral follicle count (AFC) determined before and after ovulation in 57 mares.

The number of follicles of each category was counted separately. The AFC was higher in ovaries with smaller follicles up to a diameter of 25 mm. There were a maximum of two larger follicles (≥25 mm).

Table 1 : Number (median, minimum, maximum) of antral follicles depending on their diameter.

| Diameter (mm) | 2-5 | 6-10 | 11-15 | 16-20 | 21-25 | 26-30 | 31-35 | 36-40 | >40 | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| Mares (n) | 100 | 101 | 101 | 101 | 101 | 101 | 100 | 101 | 100 | 101 |
| Median | 9 | 6 | 5 | 2 | 1 | 0 | 0 | 1 | 0 | 28 |
| Minimum | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 |
| Maximum | 37 | 21 | 17 | 7 | 5 | 2 | 2 | 2 | 2 | 59 |

The AMH ranged from 0.07 to 3.56 ng/mL with a median value of 0.59 ng/mL (Figure 3).

Three outlier AMH concentrations were measured (2.49, 2.96 and 3.56 ng/mL). The AFC of smaller follicles (diameter 2-30 mm; r = 0.58, P < 0.0001; Figure 4), but not of bigger follicles (diameter >30 mm; r = 0.04, P > 0.05) was related to AMH.

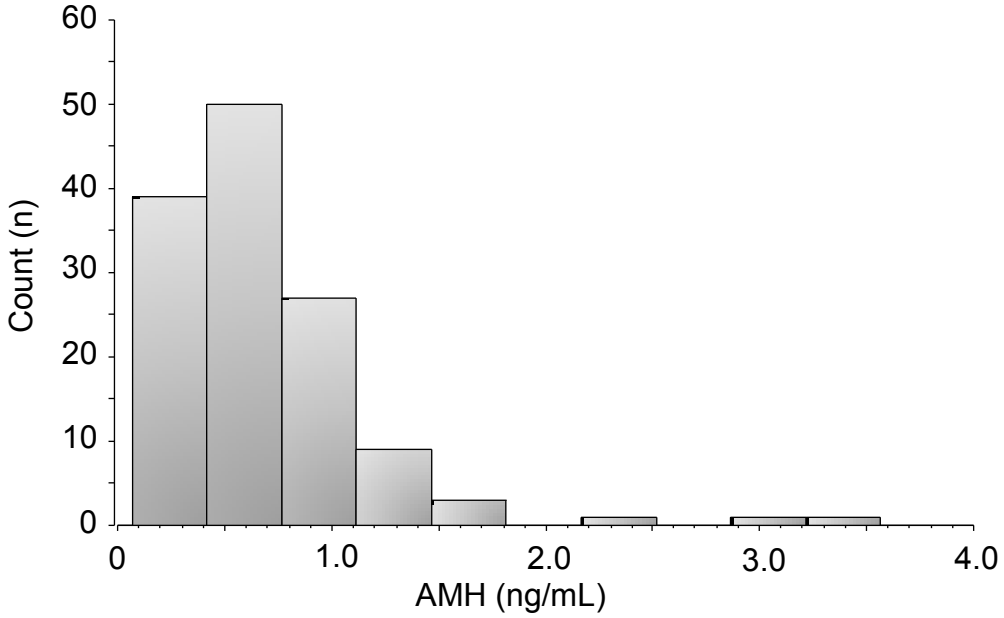


Figure 3: Serum concentration of Anti-Müllerian hormone (AMH) in 132 mares.

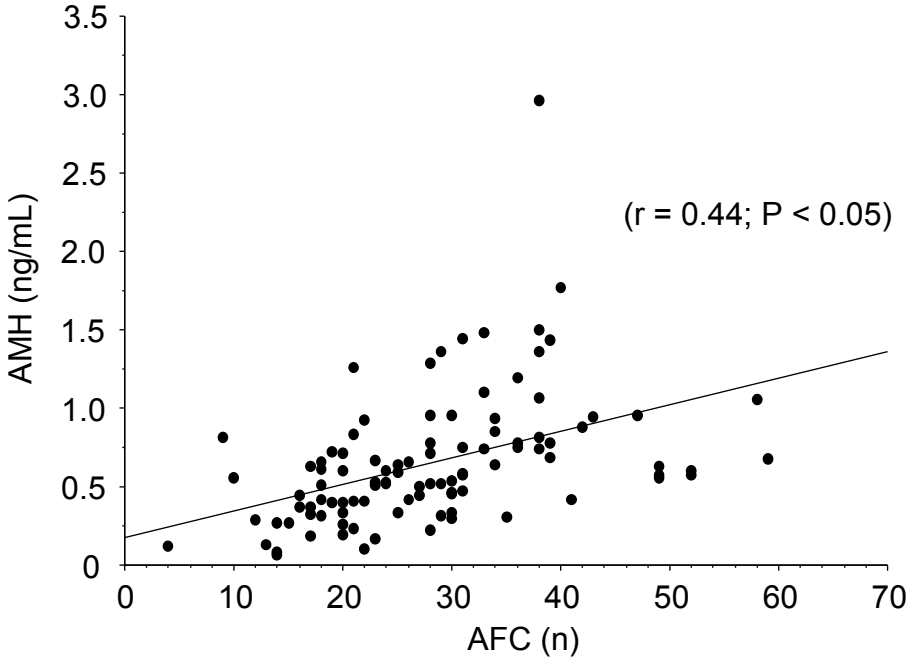


Figure 4: Relationship between the antral follicle count (AFC) and the serum concentration of anti-Müllerian hormone (AMH) in follicles with a diameter ≤30 mm.

## 5.3 Relationships between age, reproductive status, fertility, antral follicle count and serum concentration of anti-Müllerian hormone in the mares

Neither AFC (r = 0.03, P > 0.05) nor AMH (r = -0.13, P > 0.05) were related to the age of the mares. Furthermore, AFC and AMH were independent (P > 0.05) from the reproductive status of the mares (Figure 5). The AFC and AMH did not differ (P > 0.05) between mares getting pregnant or not during the season (Figure 6). Also the NCP was neither related (P > 0.05) to AFC nor to AMH (Figure 7).

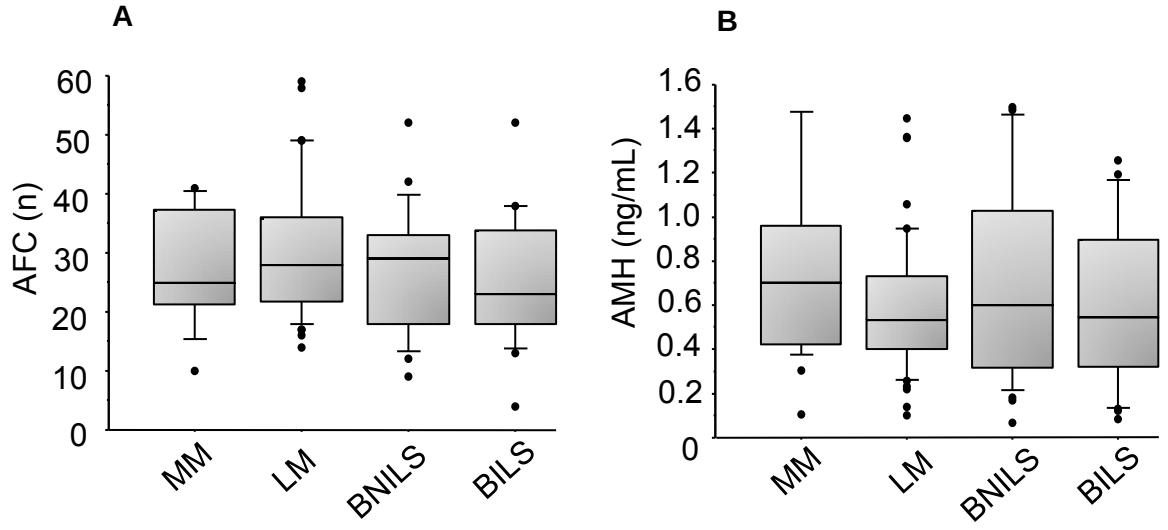


Figure 5: Antral follicle count (AFC; Fig. A) and anti-Müllerian hormone concentration (AMH; Fig. B) in maiden (MM), lactating mares (LM) and in barren mares not inseminated during the last season (BNILS) or inseminated during the last season (BILS). Values are presented as minimum and maximum values as well as box plots showing median values, 25% and 75% quartiles, 1.5 IQR and outliers.

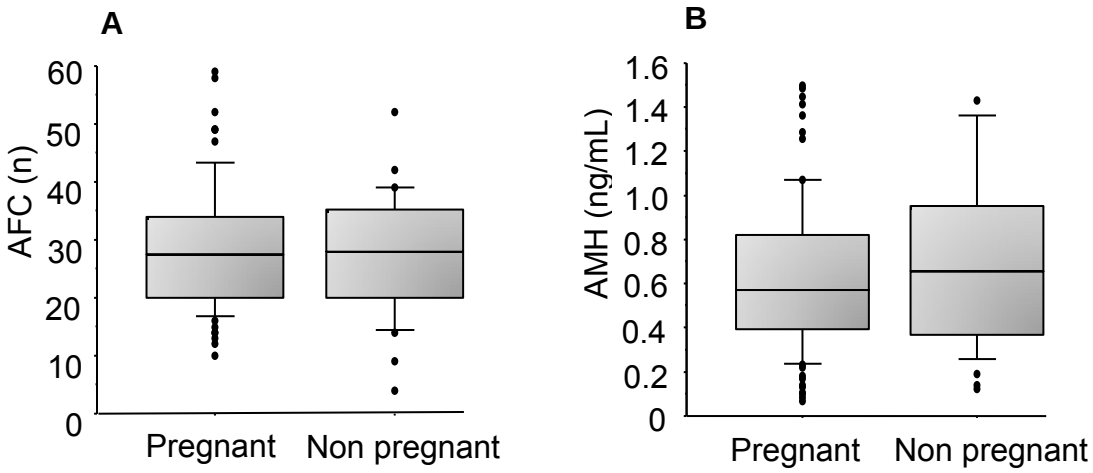


Figure 6 : Antral follicle count (AFC; Fig. A) and anti-Müllerian hormone concentration (AMH; Fig. B) in mares which got pregnant or not during the breeding season. Values are presented as minimum and maximum values as well as box plots showing median values, 25%, and 75% quartiles, 1.5 IQR and outliers.

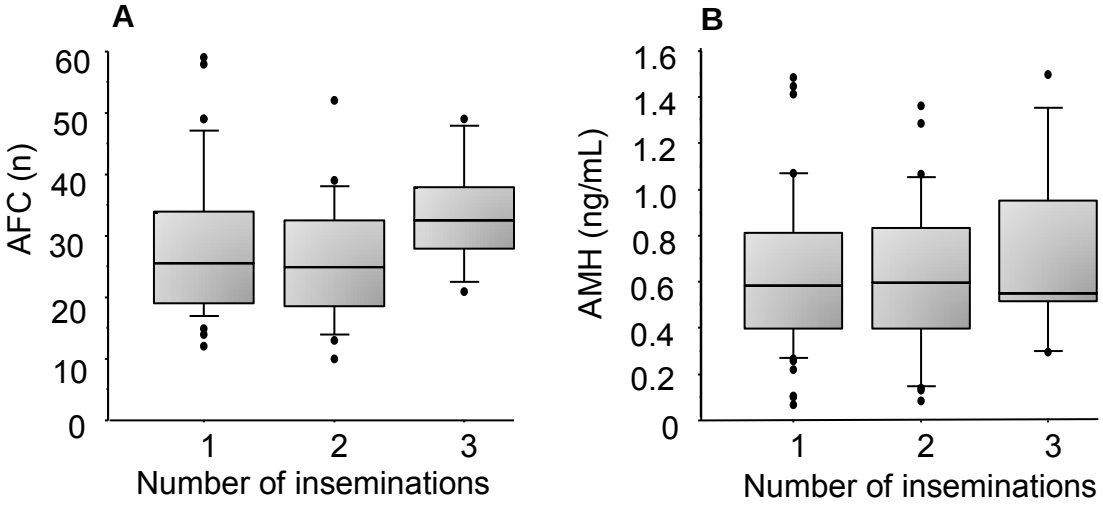


Figure 7 : Antral follicle count (AFC; Fig. A) and anti-Müllerian hormone concentration (AMH; Fig. B) in mares inseminated once (1), twice (2) or three times (3) during the breeding season. Values are presented as minimum and maximum values as well as box plots showing median values, 25%, and 75% quartiles, 1.5 IQR and outliers.

# 6 Discussion

Mares showed a strong inter-individual variability in AFC. The AFCs determined in our study were similar to those published by Claes et al. (Claes et al., 2015). Strong inter-individual differences of AFC were also described in cows (Ireland et al., 2008; Mossa et al., 2012) and in women (Gougeon et al., 1994; Scheffer et al., 1999). The AFC of follicles with a diameter up to 25 mm was higher than the AFC of bigger follicles. This phenomenon is not surprising, because deviation and selection of the dominant follicles occur at a diameter of 22.5 mm (Ginther et al., 2001; Ginther et al., 2004). As a consequence follicular growth of smaller follicles is inhibited if there is a dominant follicle.

There was a high relationship between AFC determined before and after ovulation, indicating a high reproducibility of the measurement of AFC by using two-dimensional B-mode sonography. However, surprisingly the AFC was higher after ovulation than before ovulation. It seems that the large dominant follicles compressed smaller follicles or made them less visible because the ultrasound focus was not at the appropriate depth of examination. Conventional ultrasonographic devices make images in two dimensions using ultrasound waves with a high frequency that are produced by piezoelectric crystals of the transducer, reflected from the body structures and recollected by the transducer. Artefacts are produced due to the physical principles of the emission and collection of those waves. In case of a round or oval structure such as a dominant follicle, acoustic or edge shadow may appear on the margin of the structure and in our case could hide other follicles (Kirberger, 1995). Therefore, the determination of AFC seems to be more accurate without the presence of a big follicle.

The AMH in the serum showed also a high inter-individual variability among the mares similar to those of other studies (Almeida et al., 2011; Vernunft et al., 2011). Mares with ovarian granulosa cell tumours examined in other studies (Almeida et al., 2011; Ball et al., 2008) showed distinctly higher AMH concentrations (min. 14; max 10’596 ng/mL) compared to the three mares showing outlier AMH concentrations measured in our study. This finding indicates that none of the mares of our study had ovarian granulosa cell tumours.

In our study the AFC was positively related to serum AMH. Such an association has already been described in other studies on mares (Claes et al., 2015) and other species (bovine: Ireland et al., 2008; Rico et al., 2009; mice: Kevenaar et al., 2006; human: de Vet et al., 2002; van Rooij et al., 2005). However, as already described by Claes et al. (2015) the correlation was only significant if AMH was compared with the number of follicles with a diameter between 5 and 20 mm. This was also highlighted in our study with the positive association between AMH and AFC of follicles ≤30 mm. This finding clearly refers to a higher AMH synthesis of smaller follicles in mares (Ginther et al., 2001). Similar observations were made in cows (Rico et al., 2009), mice (Kevenaar et al., 2006) and in women, where AMH was mainly associated with follicles showing a diameter up to 8 to 10 mm, i.e. before they are getting dominant (Jeppesen et al., 2013; Weenen et al., 2004). The follicle size of 8 to 10 mm in women corresponds to follicles with a size of 19 to 22.5 mm in mares (Ginther et al., 2004).

Neither AFC nor AMH were related to the age of the mares. A missing association between these parameters in mares has already been described by others (Gharagozlou et al., 2014; Vernunft et al., 2011). As already mentioned, there are tremendous inter-individual differences in the AFC between mares (Claes et al., 2015; Driancourt et al., 1982). These differences could already be observed in young mares and up to now the reason for this phenomenon is not known (Driancourt et al., 1982). In the bovine species it has been shown that environmental factors during pregnancy could affect the AFC or the AMH concentration in the offspring. Repeated elevations of the SCC in milk as an indicator of a mastitis in pregnant cows caused lower serum AMH concentrations in the offspring (Ireland et al., 2010). In addition, maternal undernutrition in cattle (Mossa et al., 2013) and sheep (Borwick et al., 1997; Da Silva et al., 2002; Rae et al., 2001) can have a negative impact on the AFC in the offspring. Assuming that such criteria can influence the AFC of mares' progeny, it would have been interesting to know the environmental conditions of the mares during the fetal and postnatal life. However, this information was not available in the mares examined in our study. Therefore, future prospective experimental studies should be carried out to investigate if the environment affects the AFC of mares like in other species. The variability in management of mares (husbandry in boxes or on pasture, in groups or as singles, usage in sport or only for breeding, feeding) is high

and might be a reason for the high inter-individual variation in AFC between mares independent from their age. In contrast to our study, other authors (Claes et al., 2015) found a significant negative correlation between AFC, AMH and age in middle aged (9-18 years) and aged (19-27 years) mares. It can be speculated that in the last mentioned study the mares were living under more similar environmental conditions compared to the mares of our study.

The SPR was neither related to the AFC nor to the AMH. We should bear in mind that in contrast to our findings AFC and AMH are biological markers of fertility and consequently markers of reproductive ageing in other species (Hendriks et al., 2005; Ireland et al., 2008; Kevenaar et al., 2006; Mossa et al., 2012; van Rooij et al., 2005). Reproductive ageing is defined in humans as a decrease in quantity and quality of the oocytes (te Velde and Pearson, 2002) and a low follicle pool leads earlier to menopause (te Velde and Pearson, 2002), to decreased IVF results (Hendriks et al., 2005), and possibly to chromosomal aneuploidies (Freeman et al., 2000). Similarly, a reduction of 50% of the follicle pool in mice by performing a hemi-ovariectomy induced a precocious infertility and aneuploidies of the oocytes (Brook et al., 1984; Eichenlaubritter et al., 1988). In contrast to those findings, our results suggest that a diminished ovarian reserve is not associated with an inferior oocyte's quality, although the quality of oocytes diminishes with age in mares. Older mares (>20 yrs) show more morphological anomalies of the oocytes (Altermatt et al., 2009) and the probability that the embryos reach the uterus is diminished in those mares (Carnevale et al., 1993). Transfer of oocytes of old mares (20-26 yrs) in oviducts of young recipients produced significantly less embryos (31% vs 92%) than transferred oocytes from young donors (6-10 yrs) (Carnevale and Ginther, 1995). In our study most of the examined mares were younger than 20 years and therefore do not belong to the category of mares showing poor oocytes quality.

An absent association between AMH and fertility has also been observed in women younger than 34 years old. In these women a reduced concentration of AMH did not predict an inferior result in an IVF program (Wang et al., 2010). However, other studies found contradictory results. In women, fertility declines from the age of 30 and is correlated with a constant depletion of the AFC as well as with the decrease of AMH. Also in cows, fertility is positively related with AFC and AMH (Cushman et al., 2009; Ireland et al., 2011; Jimenez-Krassel et al., 2009; Mossa et al., 2012; Oliveira et al., 2002). The findings of our study may indicate that in mares other factors than

AFC and AMH are more important for fertility. It has been shown in numerous studies that the uterine environment of the mare has a strong impact on the survival rate of the embryos (Carnevale and Ginther, 1992; Causey et al., 2000; Esteller-Vico et al., 2007; LeBlanc, 2008; LeBlanc and Causey, 2009; Ricketts and Alonso, 1991; Troedsson, 1999; Troedsson and Liu, 1991; Watson, 2000).

The reproductive status of the mares was neither related with the number of follicles nor with AMH. This finding was consistent to the results of a study, where AMH concentrations in lactating mares with a foal aside did not differ from those of mares without foals (Gharagozlou et al., 2014). A lactating mare is a female with a proven fertility. Therefore, following the hypothesis that a high fertility is linked with higher AFC and AMH, a lactating mare should show a higher AFC. Inversely, a barren mare having difficulties to become pregnant should show a reduced AFC and a decreased concentration of AMH. The lack of associations between reproductive status, AFC and AMH, respectively, strengthens the assumption that fertility in mares might be more affected by uterine than by ovarian disturbances. Interestingly, the last mentioned study showed that a delayed uterine clearance after insemination was associated with lower AMH concentrations in blood compared to mares with a physiological uterine clearance (Gharagozlou et al., 2014). The authors were not able to explain this association. However, according to Bromfield and Sheldon (2013), E.coli endotoxins can have a negative effect on the AFC in mice and cows. In contrast to our study, where all mares were bred by AI, the mares in the study of Gharagozlou et al. (2014) were natural mated. As natural mating could induce a stronger inflammation than AI we might have missed differences between fertile and subfertile mares. If there is an effect of an endometritis on AFC and AMH, respectively, has to be clarified in further studies.

In conclusion, there is a high variability in antral follicle count as well as in anti-Müllerian hormone concentration and both parameters are related with each other as in other species. In contrast to other species, neither AMH nor AFC in mares were related to fertility after artificial insemination.

# 7 Literature

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